UNIVERSITI PUTRA MALAYSIA

MOLECULAR ANALYSIS OF *DICHELOBACTER NODOSUS* ISOLATED FROM FOOTROT INFECTED SHEEP IN MALAYSIA

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By

ZUNITA BINTI ZAKARIA

Thesis Submitted in Fulfilment of the Requirement for the Degree of Doctor of Philosophy in the Faculty of Veterinary Medicine Universiti Putra Malaysia

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Chairman: Professor Dato' Dr. Sheikh Omar Abdul Rahman B.V.Sc. (Queensland), M.V.Sc. (Saskatchewan), M.R.C.V.S. (London)

Faculty: Veterinary Medicine

Footrot has become an increasingly important disease of sheep in Malaysia. Therefore, the molecular analysis of the causative agent of footrot, *Dichelobacter nodosus* isolated from footrot infected sheep was undertaken. Fifteen *D. nodosus* isolates were recovered from 38 sheep showing clinical signs of footrot in two government sheep farms located approximately 200 km apart. The isolates were studied and results analysed. Preliminary identification of the organism was carried out by the Gram-stain method while the polymerase chain reaction (PCR) method using species-specific

primers, A and Ac, was employed for species confirmation. All 15 isolates produced a single product of approximately 780 basepairs. Although obtained from two different locations, all isolates were found to be of serogroup B. Two conventional methods, namely the elastase and gelatin-gel tests, were used to assess the virulence of the isolates. Generally, the isolates exhibited variations in the laboratory characteristics. Based on the virulence assessment, some of the isolates appeared to have the capability for causing virulent footrot but were isolated from sheep that did not show clinical signs of the virulent form of footrot. This was probably due to the constant topical treatment regime and the vaccination programme practised by the farm management which may have caused the bacteria to not fully express its virulence characteristics.

Analysis of the fimbrial subunit gene sequence revealed the local strains had sequences that are distinct from the prototype strains. There were 94 to 97 percent amino acid similarities (identities and conserved changes) between the local isolates and the prototype strains. The expression of *D. nodosus* fimbriae serotype B2 in an easily grown aerobe, *Pseudomonas aeruginosa*, were carried out successfully. *Dichelobacter nodosus* fimbrial subunit gene was cloned in an expression vector, pUCpKS downstream the *lac* promoter to construct the recombinant plasmid

pMAL99. Recombinant *P. æruginosa* cells containing this construct were able to produce a high yield of fimbriae. The fimbriae were physically, structurally and antigenically indistinguishable from those produced by the *D. nodosus* isolates from which the fimbrial subunit gene was originally derived. This was shown and confirmed by Western blot analysis. When the fimbriae produced by the *P. æruginosa* harbouring pMAL99 were extracted, purified and used as vaccines in sheep, the results conclusively showed that these vaccines were equally effective as either the native whole cells or isolated fimbriae from *D. nodosus* in eliciting the antibody response. The vaccinated sheep were found protected against homologous serogroup challenge. The recombinant fimbriae also produced cross-protective antibodies to heterologous serotypes B3 and B4 infections. Therefore, the monovalent serogroup specific recombinant vaccine has a good potential for use in farms in this country to protect sheep against footrot.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Doktor Falsafah

ANALISIS MOLEKUL *DICHELOBACTER NODOSUS* YANG DIPENCILKAN DARI BEBIRI YANG MENGHIDAP REPUT KAKI DI MALAYSIA

Oleh

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Penyakit reput kaki adalah penyakit yang semakin penting di Malaysia. Oleh itu, analisis molekul ke atas bakteria penyebab penyakit reput kaki, *Dichelobacter nodosus* yang dipencilkan dari bebiri yang menghidap penyakit reput kaki telah dijalankan. Lima belas isolat *Dichelobacter nodosus* telah dipencilkan dari 38 ekor bebiri yang menghidap penyakit reput kaki dari dua ladang kerajaan yang terletak lebih kurang 200 km di antara satu sama lain. Pencilan tersebut dikaji dan dianalisis. Diagnosis telah berjaya dilakukan melalui kaedah pewarnaan Gram sementara reaksi

polimerase rantai (PCR) dengan menggunakan primer spesifik, A dan Ac telah digunakan untuk konfirmasi spesis. Kesemua isolat didapati menunjukkan reaksi positif dalam kaedah PCR dengan menghasilkan satu produk 780 pasangan bes. Walaupun semua isolat dipencilkan dari dua lokasi yang berbeza, semuanya adalah dari satu serogroup B. Dua kaedah konvensional iaitu, ujian elastase dan gel-gelatin telah digunakan untuk menentukan tahap kevirulenan isolat. Secara amnya, isolat menunjukkan variasi dalam ciri-ciri kevirulenan di dalam makmal. Sesetengah isolat didapati mempunyai keupayaan menyebabkan tahap penyakit yang virulen, tidak menunjukkan tanda klinikal yang sedemikian. Ini mungkin disebabkan oleh rawatan topikal yang diamalkan oleh pihak pengurusan ladang dan mungkin juga hasil dari program vaksinasi yang menyebabkan bakteria tidak menunjukkan tahap kevirulenan sebenar.

Analisis jujukan gen fimbria menunjukkan isolat tempatan mempunyai jujukan yang berbeza dari strain prototaip. Terdapat 94 hingga 97 peratus persamaan (identiti dan penggantian konserve). Ekspresi fimbria serotip B2 *D. nodosus* di dalam bakteria aerobik, *Pseudomonas aeruginosa* telah berjaya dilakukan. Gen fimbria *D. nodosus* telah diklonkan ke dalam vector ekspresi, pUCpKS selepas kedudukan promoter *lac* untuk membentuk plasmid rekombinan pMAL99. Sel-sel rekombinan *P. aeruginosa* yang

mengandungi plasmid ini didapati menghasilkan fimbria yang sama dari segi fizikal, struktur and antigenisiti dengan fimbria asal *D. nodosus*. Ini telah ditunjukkan dan dibuktikan dengan analisis pemblotan Western. Fimbria yang dihasilkan dari *P. aeruginosa* yang membawa pMAL99 diekstrak, ditulenkan dan digunakan sebagai vaksin kepada bebiri. Keputusan menunjukkan vaksin ini mempunyai tahap efektif yang setara dengan sel penuh *D. nodosus* ataupun fimbria yang dipencilkan dari *D. nodosus* itu sendiri dalam menginduksikan respons antibodi. Bebiri yang divaksinasi adalah dilindungi dari cabaran dari serogroup homologus. Fimbria rekombinan ini juga didapati menghasilkan perlidungan silang kepada infeksi dari serogroup heterologus B3 dan B4. Oleh itu, terdapat potensi yang baik untuk mengaplikasi vaksin serogroup spesifik di ladang tempatan untuk melindungi bebiri daripada penyakit reput kaki.

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I certify that an Examination Committee met on 12th December 2001 to conduct the final examination of Zunita binti Zakaria on her Doctor of Philosophy thesis entitled "Molecular Analysis of *Dichelobacter nodosus* Isolated from Footrot Infected Sheep in Malaysia" in accordance with Universiti Pertanian Malaysia (Higher Degree) Act 1980 and Universiti Pertanian Malaysia (Higher Degree) Regulations 1981. The Committee recommends that the candidate be awarded the relevant degree. Members of the Examination Committee are as follows:

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DECLARATION

I hereby declare that the thesis is based on my original work except for quotations and citations, which have been duly acknowledged. I also declare that it has not been previously or concurrently submitted for any other degree at UPM or other institutions.

(ZUNITA BINTI ZAKARIA)

Date: 8/1/2002

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF NOTATION

Amino acid	one-letter notation
alanine	A
arginine	R
asparagine	N
aspartate	D
cysteine	C
glutamate	E
glutamine	Q
glycine	G
histidine	H
isoleucine	I
leucine	L
lysine	K
methionine	M
phenylalanine	F
proline	P
serine	S
threonine	T
tryptophan	W
tyrosine	Y
valine	V

CHAPTER 1

GENERAL INTRODUCTION

Footrot is a contagious disease of ruminants, particularly sheep and goats although cattle and deer may also be affected. It is present worldwide and has a significant economic impact in sheep farming countries with a temperate climate and a moderate to high rainfall, such as Australia and New Zealand (Stewart, 1989). It is responsible for serious losses to the sheep industry in reduced meat and wool production, lowered fertility, and the high costs of labour and materials used in treating affected animals (Stewart *et al.*, 1984; Marshall *et al.*, 1991; Glynn, 1993). The disease is characterized by inflammation of the interdigital skin and hoof matrix leading to an underrunning and separation of the hoof from the epidermal tissues. Classical signs of footrot are severe lameness and pain with the infected animal preferring to walk on its knees when only the front feet are affected, or lying prone when all four feet are affected (White, 1991). The increasing awareness of animal welfare issues associated with footrot has brought the disease more into the forefront of the industry.

The main etiological agent of footrot is *Dichelobacter nodosus* formerly known as *Bacteroides nodosus* (Dewhirst *et al.*, 1990). It is a Gram-negative, strictly anaerobic, nonsporeforming bacteria. A culture of *D. nodosus* deposited in the American Type Culture Collection as accession no. 25549 has been designated the prototype strain (Skerman, 1989). *Dichelobacter nodosus* lives only in diseased hooves and survives no longer than 7-14 days in faeces, soil or pasture.

Footrot has become an increasingly important disease of sheep in Malaysia. Even though the disease is known to exist for the last two centuries in many parts of the world, it has only been detected in this country in the last six years (Yii, 1995). The first confirmed case of footrot in Malaysia occurred in a government sheep farm namely the Institut Haiwan Kluang, Johor; in the southern part of Peninsular Malaysia in 1994. The disease is now present in other farms throughout the country. Importations of sheep were made from diverse countries such as Australia, Brazil and Thailand which might have brought the disease into the country.

Vaccines containing representative strains from all major serogroups incorporated into oil-based adjuvant have been available since 1981. They consist of a mixture of killed cultures of different serogroups (Walker, 1988). The conventional vaccines are very costly (Mattick

and Hobbs, 1990), thus, a genetically engineered vaccine was developed. The fimbrial gene from prototype *D. nodosus* strains has been cloned and expressed as extracellular fimbriae in an easily grown aerobe, *Pseudomonas aeruginosa.* Fimbriae extracted from the recombinant *P. aeruginosa* cells were used as vaccines against *D. nodosus* infection.

Sheep farms in Malaysia started to use the commercial footrot multivalent killed whole cells vaccines in late 1996. The vaccination did reduce the prevalence of footrot but the strength and duration of the immunity achieved was limited. The short duration of the immunity is associated with a poor agglutinin response in animals receiving multiple fimbrial antigens of *D. nodosus* simultaneously. Moreover, a previous study on local *D. nodosus* isolates found them to be antigenically different from the prototype strain (Zunita, 1998). There is now a recurrence of the disease which has become worse under conditions favouring the infection.

Therefore an extensive study is needed to obtain a comprehensive knowledge about the etiological agent and to understand the situation of the infection and the disease in the country. Conventional eradication methods practised in the temperate countries are unlikely

to succeed in Malaysia where the weather is warm and humid throughout the year. The findings of the study will enable the formulation of suitable measures to control footrot.

The objectives of this study were:

(1) to isolate, identify and assess the virulence of *D. nodosus* from clinical cases of footrot in sheep kept in farms in Malaysia.

(2) to determine the relationship between fimbriae from different strains in Malaysia and the prototype strains by analysing the nucleotide as well as the amino acid sequences of the fimbrial subunit gene.

(3) to determine the possibility of producing a serogroup specific recombinant vaccine by cloning and expressing local *D. nodosus* fimbrial subunit gene in an aerobe surrogate host, *Pseudomonas aeruginosa*.

(4) to investigate the antigenic properties of a recombinant vaccine prepared from fimbrial genes of a local isolate in sheep.